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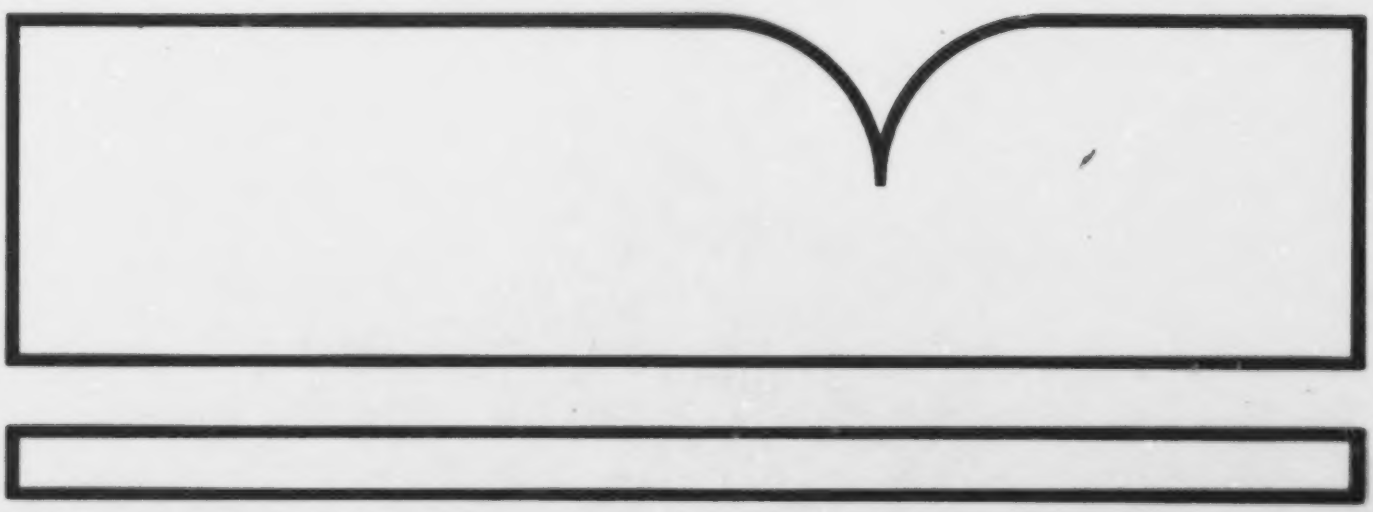

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THE EXCRETION OF PESTICIDES IN SALIVA AND ITS VALUE IN ASSESSING EXPOSURE

Key Words: Pesticides, Saliva, Toxicity, Kepone, Carbaryl

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ABSTRACT

The possibility that pesticides are secreted in the saliva and that this secretion can be used to assess exposure to pesticides is discussed. Both Kepone and carbaryl were tested through a series of *in vitro* studies and *in vivo* experiments including both acute and subchronic studies. In the *in vivo* studies, both Kepone and carbaryl were detected in the saliva for the first twenty-four hours, but seldom thereafter. Analysis of urinary samples indicated a rapid clearance of the substances from the animals. *In vitro* uptake and efflux studies suggested that movement of both substances was by passive diffusion and

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reached a plateau after 60 minutes of incubation. Effux rates were unaffected by experimental conditions, but were ten times faster for carbaryl.

INTRODUCTION

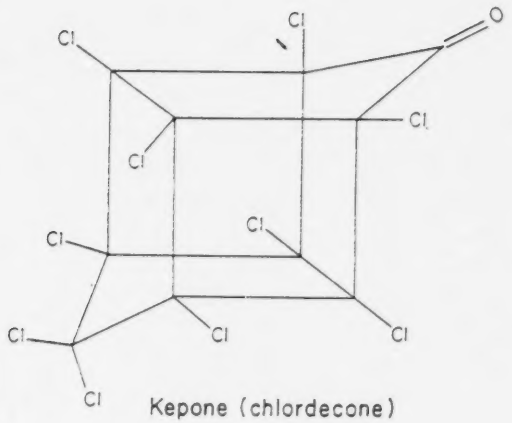
The buccal cavity, including soft tissue, hard tissue, and salivary fluid, is often overlooked as site of toxicity. Lesions such as gingival hyperplasia and lingua nigra (black hairy tongue) have been reported following protracted use of diphenylhydantoin and penicillin, respectively. This suggests that drugs may be excreted in the saliva and alter the physiology of the mouth. Afonsky[1] reported a number of agents present in saliva including drugs and heavy metals. Until recently, salivary fluid has been investigated primarily as an aid to deglutition, tasting, and digestion, as an inhibitor of bacterial activity, and as a modulator of caries development. The use of saliva as an aid in diagnosis has been mentioned by Joselow et al,[2] but thus far has not been adequately evaluated.

During the past 10 years, this laboratory has been investigating the mechanism by which drugs and other chemicals are excreted or secreted in saliva. A number of *in vitro* procedures have been developed, and their reliability established. The mechanism of movement across the salivary membrane and the biotransforming capabilities of salivary tissue have been determined for a number of chemicals, including antibiotics,[3] sulfonamides

and barbiturates,[4] salicylic acid,[5,6,7] nicotine,[8] diphenylhydatoin,[9,10] and tetrahydocannibinol.[11,12]

In 1976, in Hopewell, Virginia, the inadvertent contamination of workers and the environment with Kepone revived concern for developing new, non-invasive techniques to monitor pesticide exposures. Previous experience demonstrated that a variety of compounds were secreted in saliva. Saliva might provide as a useful diagnostic fluid. In addition, the collection of saliva would be better tolerated than the collection of blood or urine. Studies were conducted to verify this hypothesis. Representatives of the three chemical classes of insecticides (chlorinated hydrocarbons, carbamates, and organo-phosphates) and one herbicide (2,4 D) were chosen for the initial study. Kepone and carbaryl will be discussed.

Kepone (decachlorooctahydro-1,3,4 methano-2H-cyclobuta (ce) pentalen-2-one), also known as chlordecone (Figure 1), is a complex polycyclic chlorinated insecticide of high stability (registered tradename-Kepone® - Allied Chemical Corporation, Morristown, New Jersey). It is useful in the control of insects, including cockroaches,[13] rust mites, wireworms, and root borers. Contamination of the environment with Kepone may pose adverse effects on both terrestrial and aquatic life. Kepone adversely affects reproduction in quail,[14] and mice,[15,16] decreases egg production in laying hens,[17] and induces fetal toxicity in rats.[18] Kepone has also been identified as the agent responsible



Kepone (chlordecone)

Figure 1

The Structure of Kepone

for neuropathological signs and symptoms, liver damage, and sterility in male workers involved in its production.[19] The absorption, distribution, and biotransformation of Kepone in the male rate have been reported by Matthews & Morales[20] and Egle et al.[21] High levels of Kepone have been detected in the liver, adipose tissue, feces, and urine of man[22-24] and metabolites of chlordecone have been recently reported by Blanke et al.[22]

Carbaryl (1-naphthyl-N-methyl-carbamate, Sevin(R)) is recommended as a broad spectrum pesticide and is widely used due to its comparatively low mammalian toxicity.[26] Carbaryl exhibits adverse side-effects on the reproductive fitness of the rat and gerbil,[27] and on the neuromuscular apparatus of swine,[28] and on thermo-regulation in the mouse.[29] Distribution and metabolism

of carbaryl have been reported by Dorough and Cassida,[30] Leeling and Cassida,[31] and reviewed by Casper and Pecas.[32]

The present investigation was designed to determine if analysis of saliva could provide a quick and easy method to assess exposure to Kepone and carbaryl. *In vivo* and *in vitro* studies were conducted to identify the mechanism of transport of these insecticides into saliva.

MATERIALS AND METHODS

Chemicals

^{14}C-carbaryl (1-naphthyl-n-methyl-(^{14}C)-carbamate) having a specific activity of 59.5 μCi/mmole was obtained from Amersham/Searle (Arlington Heights, Ill.). Unlabeled carbaryl was obtained from Union Carbide and was verified by thin layer and gas chromatography. ^{14}C-Kepone (Pathfinders Laboratories, St. Louis, MO) with a specific activity of 19.38 μCi/mmole was used. All other chemicals were reagent grade unless otherwise stated.

Uptake and Efflux Experiments

The *in vitro* procedure used for uptake and efflux experiments has been reported by Putney and Borzelleca.[7,8] The submaxillary glands were isolated and removed by blunt dissection from anesthetized rats and cut into slices weighing 30-80 mg. Krebs-Ringer-Tris (KRT) buffer, pH 7.4, used as the incubation medium, was maintained at 37°C (unless otherwise stated) and constantly aerated with 100% O_2. Slices were equilibrated for

60 minutes and then incubated with ^{14}C-Kepone or ^{14}C-carbaryl for 60 minutes. Dinitrophenol (10^{-3}M), potassium cyanide (10^{-3}M), nitrogen, and lowered temperature (5°C) were used as inhibitors. Oxygen uptake was measured on a YSI model 53 oxygraph.

Efflux rates were determined using a modification of the isotope washout method of Putney and Borzelleca.[7,8] After the 60 minute equilibration period, the tissue slices were transferred to incubation vials containing 10^{-5}M, 10^{-4}M, 10^{-3}M ^{14}C-Kepone or ^{14}C-carbaryl for another 60 minutes. The tissues were then successively transferred through a series of 25 vials, each containing 5 ml. KRT buffer for 20min digestion in NaOH. The data were expressed as a percentage of radioactivity remaining in the tissue slice at each point in time. The results were plotted logarithmically to yield desaturation curves.[33]

Acute Studies

Male, albino Sprague-Dawley rats (Flow Labs) weighing between 225-350 grams were anesthetized (60 mg/kg sodium pentobarbital, i.p.) and the femoral artery cannulated with P.E. 20 tubing. A 1.5 cm incision was made through the abdominal wall to locate the stomach. Carbaryl, dissolved in corn oil, was injected directly into the stomach to avoid contamination of the saliva samples. Rats were dosed with 0, 50, 100, or 200 mg/kg carbaryl. Blood and saliva samples were collected at 0, 15, 30, 60, 90, 120, 240, and 360 minutes after dosing. When possible, tears were also collected at

these time points. Salivary flow was stimulated with pilocarpine (20 mg/kg) in both experimental and control animals. Cholinesterase activity was determined,[34] and protein concentration measured.[35] The presence of carbaryl and its metabolites in these fluids was determined by TLC.[30] Red blood cell (RBC) counts were performed on a Coulter Counter Model ZBI. Kepone was administered at 50 mg/kg and the samples collected at 1, 2, 4, 6, 24, and 48 hours after administration. The biotransforming capability of the rat salivary gland for Kepone was assessed using thin-layer chromatography (1:1, diethylether: hexane)[20] and for carbaryl using the method of Dorough and Cassida.[30]

Subchronic Study

Fifty male, albino Sprague-Dawley rats (Flow Labs) mean weight 250g, were divided into 5 groups of ten rats each. Carbaryl was dissolved in Mazola(R) corn oil, and the rats were gavaged daily with either 0, 1, 10, 100, or 200 mg/kg carbaryl for 13 weeks. Food and water were available ad libitum throughout the study. Weights were recorded weekly. Each week, two rats from each group were anesthetized with ether, and blood and saliva were collected. The presence of carbaryl and its metabolites in these fluids was determined by thin layer chromatograph (TLC) and/or gas chromatography. Cholinesterase activity in blood, plasma, and saliva was determined at the end of exposure.[34]

RESULTS

In Vivo Studies

The levels of carbaryl in saliva and plasma appear to be parallel over time (Figure 2). Carbaryl reaches its highest level in both plasma and saliva at 60 minutes after administration and subsequently declines.

During the thirteen week study, neither carbaryl nor α-naphtol could be detected in either plasma or saliva 24 hours after dosing. In addition, the red blood cell (RBC), plasma, and salivary cholinesterase activity could not be differentiated from controls at 24 hours. However, a variety of metabolites were found in urine,

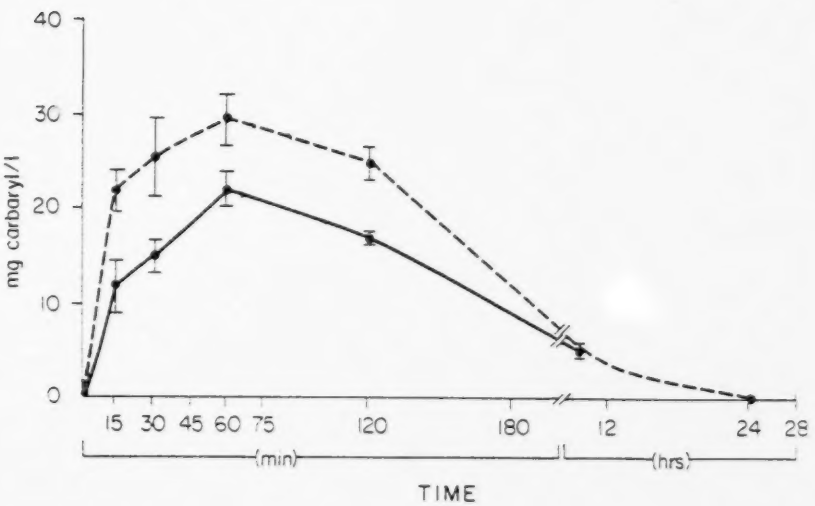


Figure 2. The concentration of carbaryl in saliva and plasma. Carbaryl was administered as a single dose 200 mg/kg directly into the stomach. Saliva _____, Plasma ----------

indicating a rapid clearance of carbaryl from the rat. This confirms the data of Casper and Pekas[32] who demonstrated with ^{14}C-carbaryl that 97.1% of the radioactivity administered was recovered as $^{14}CO_2$ within 2 hours of oral dosing, and 100% within 24 hours.

Figure 3 represents a composite of chromatograms of blood, saliva, tears, and urine from rats that received carbaryl at doses of 50, 100, and 200 mg/kg. Carbaryl and α-naphthol were used as standards and other ether-extractable metabolites were designated A to I in accordance with Dorough and Cassida.[30] Results obtained with urine were similar to those reported by Dorough and Cassida[30] and Leeling and Cassida.[31] Tears (containing no blood) were found to contain carbaryl and α-naphthol at all levels. At a dose of 200 mg/kg, an unknown metabolite, designated as H-2, was detected. Metabolite D (identified as 1-hydroxy-5,6-dihydroxy-1-naphthyl methylcarbamate were found at all levels.[31] At the 50 mg/kg level, the amount of metabolite B increased in both blood and saliva, while only small amounts of D were found. Neither B nor D were detected in tears.

The effects of carbaryl and its metabolites on the cholinesterase activity of saliva and blood are demonstrated in Figure 4. There does not appear to be a dose dependent effect on cholinesterase inhibition.

Kepone is excreted in the saliva of rats following oral administration, the levels of Kepone in saliva being consistently

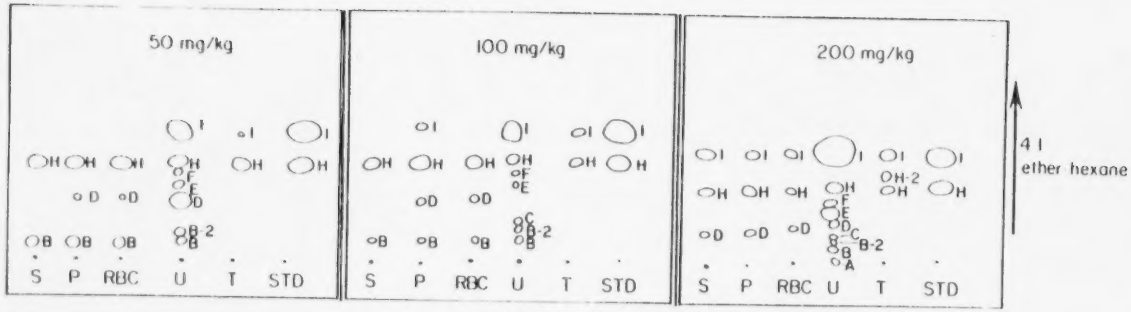


Figure 3

Composite representing metabolites detected by thin layer chromatography after cosing with 50, 100 or 200 mg/kg of carbaryl. Tissues sampled were saliva (S), plasma (P), red blood cells (RBC), urine (U), tears (T). Metabolites were designated A to I, with (α-naphthol) and H (carbaryl) used as standards (STD).

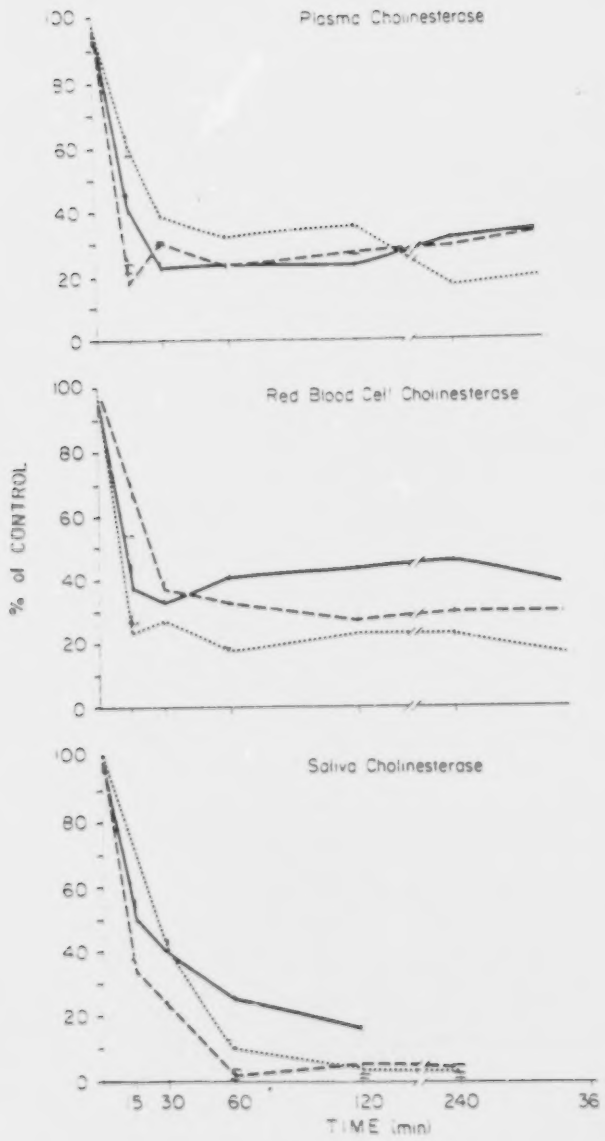


Figure 4

Effects of carbaryl on plasma, red blood cell and salivary cholinesterase. Fluids were sampled at 0,15,30,60,120,240 and 360 minutes. Carbaryl was injected directly into the stomach at 50 mg/kg .... 100 mg/kg ----, and 200 mg/kg _____, respectively.

lower than the levels in plasma (Table 1). The peak level of Kepone in saliva is reached between 6 and 24 hours. Radioactivity was also detected in the submaxillary, parotid, and sublingual salivary tissues. These levels are low when compared to levels of Kepone in liver and adipose tissue.[21] Since the S/P ratios are less than 1 throughout the experimental period, Kepone does not appear to be actively concentrated by the salivary glands.

In Vitro Uptake and Efflux Studies

Uptake of both ^{14}C-Kepone and ^{14}C-carbaryl reached a plateau after 60 minutes of incubation and was unaffected by increasing concentration, as illustrated in Figure 5. Similarily, the uptake of Kepone or carbaryl was not affected by lowering the temperature, exposure to metabolic inhibitors or lack of O_2 as demonstrated by Kepone data (Figure 6). This suggests passive diffusion of Kepone and carbaryl into the tissue slice.

A typical experiment measuring efflux rates is presented in Figure 7. After tissue incubation of 60 minutes in ^{14}C-Kepone (10^{-4}M), the slice was transferred successively to 25 vials at

TABLE 1

RATIOS OF ACTIVITY (SALIVA/PLASMA)
FOLLOWING THE ORAL ADMINISTRATION OF ^{14}C-KEPONE

	Time in Hours					
	1	2	4	6	24	48
S/P	0.07	0.09	0.17	0.29	0.30	0.02

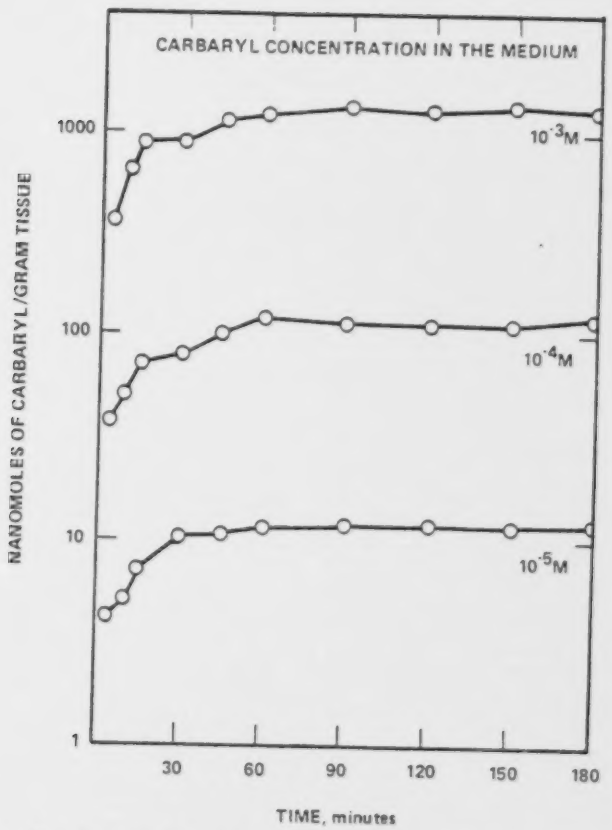


Figure 5. The uptake of carbaryl by rat submaxillary gland slices. Slices were rested for an hour prior to uptake. The time on the abscissa refers to the time after transferring the slice to the radioactive medium. Each point represents the mean of six determinations.

2 minute intervals. The rate constant for the slow component was determined by linear regression to be 0.0037 $\pm$ 0.0008 min^{-1} for the 10-50 minute period. The fast component was obtained by subtracting the extrapolated values of the slow component from

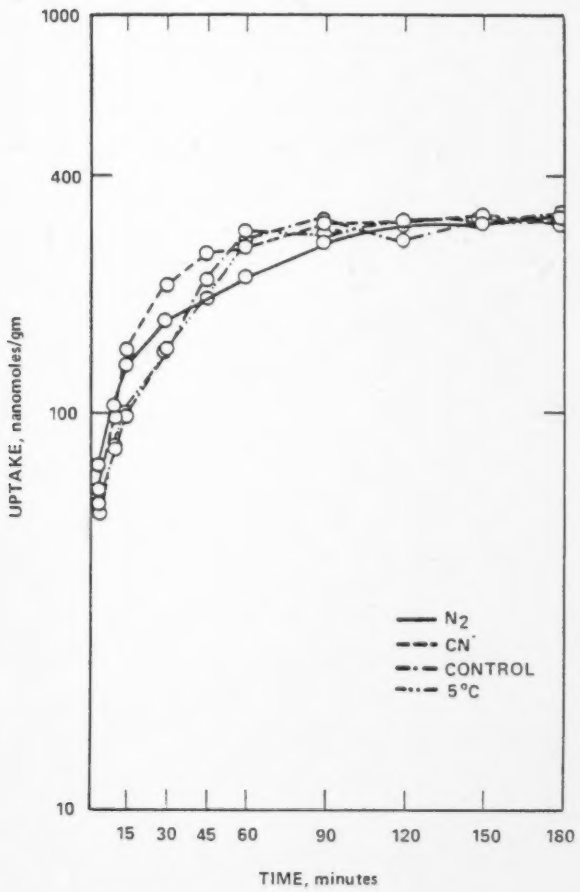


Figure 6. The uptake of Kepone (10^{-4}M) by rat submaxillary slices. Slices were rested for an hour prior to uptake. The time on the abscissa refers to the time after transferring the slice to the radioactive medium. Each point represents the mean of four determination.

the experimental points for the period 0-8 minutes. The rate constant of the fast component is 0.843 ± 0.150 min $^{-1}$.

The effects of varying conditions during the washout procedure of Kepone are shown in Table 2. No significant changes in rates

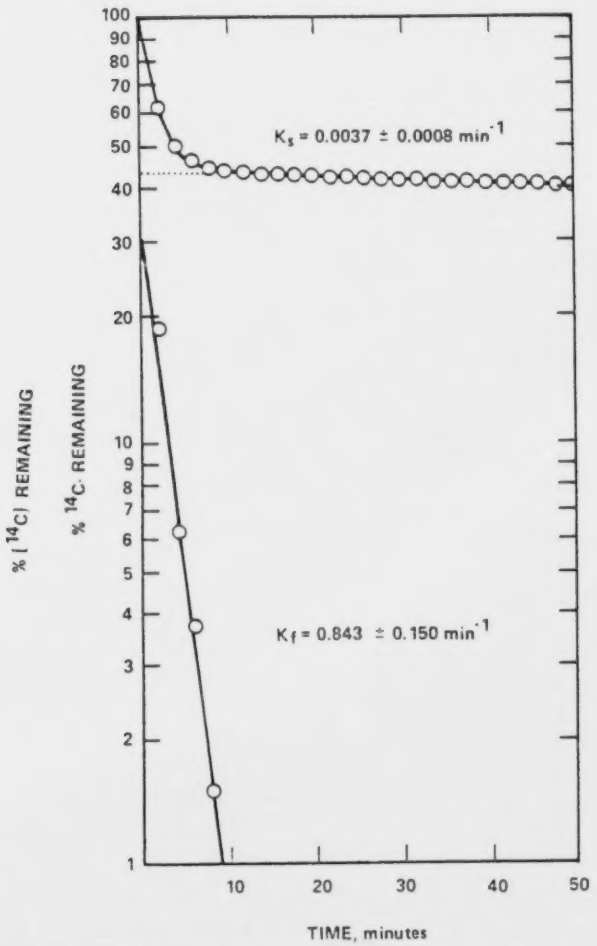


Figure 7. Efflux of ^{14}C-Kepone from a submaxillary gland slice. The slice was equalibrated for 1 hour, incubated with ^{14}C-Kepone (10^{-5}M) for 60 minutes and then washed out for 50 minutes at 2-minute intervals. The broken line represents the extrapolation of the slow component to zero time. The fast component was obtained by subtracting the values on the extrapolated line from the corresponding experimental values.

TABLE 2

Influence of Various Factors on the Rate of ^{14}C-Kepone Efflux at Three Concentrations

Condition of Incubation	10^{-3}M kepone nanomoles/gm/min		10^{-4}M kepone nanomoles/gm/min		10^{-5}M kepone nanomoles/gm/min	
	k_s	k_f	k_s	k_f	k_s	k_f
Control[a]	-0.0045 ±0.0004	-0.843 ±0.150	-0.0037 ±0.0010	-0.843 ±0.154	-0.0039 ±0.0008	-0.719 ±0.165
N_2	-0.0047 ±0.0003	-0.878 ±0.160	-0.0038 ±0.0021	-0.828 ±0.131	-0.0029 ±0.0003	-0.702 ±0.113
5°C	-0.0045 ±0.0004	-1.103 ±0.290	-0.0056 ±0.0010	-0.882 ±0.104	-0.0048 ±0.0011	-0.963 ±0.101
Cynaide (10^{-3}M)	-0.0046 ±0.0004	-0.871 ±0.180	-0.0042 ±0.0003	-0.803 ±0.110	-0.0048 ±0.0003	-0.855 ±0.040
2,4-dinitrophenol (10^{-3}M)	-0.0043 ±0.0010	-0.652 ±0.103	-0.0046 ±0.0012	-0.738 ±0.180	-0.0046 ±0.0010	-0.653 ±0.155

[a]Control conditions: pH 7.4, 37°C, aeration with 100% O_2

of efflux of Kepone from the intracellular or extracellular compartments were observed under any of the conditions employed. The efflux data for carbaryl (Figure 8) indicates a similar situation with no changes in rate observed with differing experi-

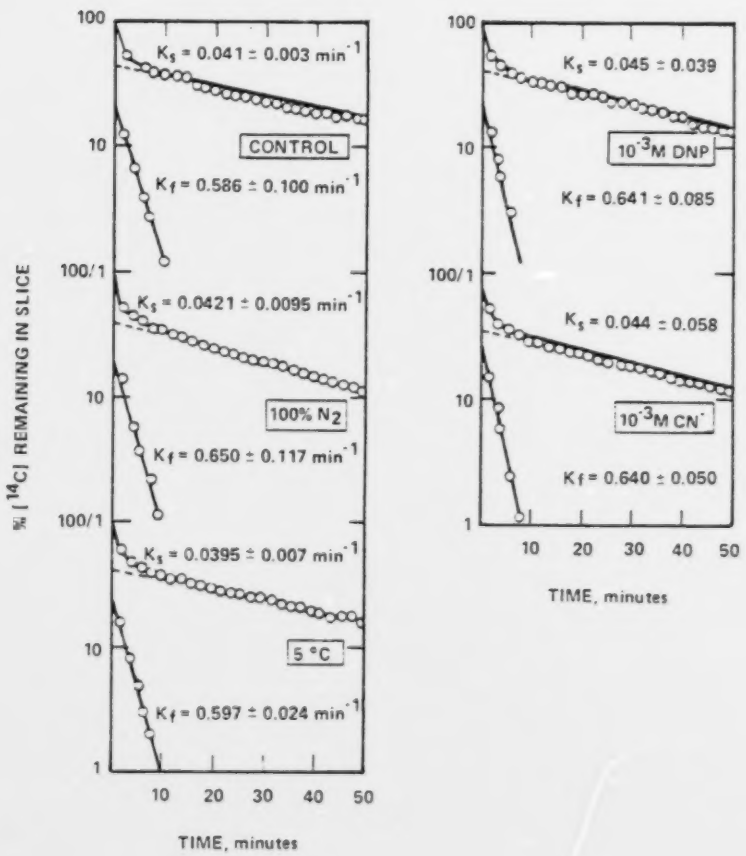


Figure 8. Efflux of 10^{-4}M ^{14}C-carbaryl by rat submaxillary gland slices with the addition of metabolic inhibitors. (100% N_2, 5°C, 10^{-3}M DNP or 10^{-3}M CN^-). Each point represents the mean of six determinations.

mental conditions. Neither carbaryl nor Kepone had any effect on O_2 consumption of these slices. These data suggest that the uptake and efflux of carbaryl within the submaxillary gland are not energy dependent.

One major difference between Kepone and carbaryl is evident in the efflux results. The ks of Kepone (10^{-4}M) is 0.0037 $\pm$ 0.0008 $^{-1}$ min., while the Ks for carbaryl (10^{-4}M) is 0.041 $\pm$ 0.0003 $^{-1}$ min. This indicates that the wash-out rate for Kepone is about ten times slower than the rate for carbaryl.

Biotransformation By Salivary Tissue

The biotransformation of ^{14}C-Kepone by homogenates of salivary glands in incubation media was determined. Only unchanged ^{14}C-Kepone was detected in both tissue homogenates and the incubation media. Either no biotransformation of ^{14}C-Kepone was occurring or the concentration of the metabolic products formed was too low to be detected with the system employed.

DISCUSSION

The uptake and efflux of carbaryl from salivary gland slices has been demonstrated to be a passive process. This also appears to be the case in the whole animal, for the metabolites that appear in the blood are also present in saliva and the concentration of carbaryl excreted in saliva in similar to the concentration present in the plasma (Figure 3). Data from time-course thin layer chromatographic separation of metabolites indicate that α-naphthol and metabolites B and D also enter the saliva in

a manner similar to carbaryl, each appearing in plasma and saliva at approximately the same time concentration.

In the rat, the activity of the cholinesterases of saliva, plasma and RBC's was inhibited by exposure to carbaryl. The inhibition of salivary esterases appeared to parallel those of the plasma and red blood cells. The esterases in rat saliva have not been well characterized. In man, however, the man salivary esterases are not cholinesterases but carboxylesterases,[36] enzymes not found in normal human serum.[37] It has also been suggested that most of the non-specific esterase activity in human saliva is derived from oral bacteria.[38] It has been demonstrated that the esterase activity of human parotid saliva accounts for only 1.5% of the activity in whole saliva.[39] The contribution of bacterial esterases to the total activity of rat saliva is not known.

An inherent problem of using cholinesterase as diagnostic tool is the reversible nature of the carbamylated enzyme. This makes the assay sensitive to both concentrations of inhibitor and substrate. It may be assumed that these competitive interactions will vary with the particular enzyme. Watts and Wilkinson[40] suggested that conformational changes at the active site of the enzyme may explain the differences in recovery rates of dimethyl and monomethyl carbamylated enzymes. Not only concentration, but also a specific chemical inhibitor can change the sensitivity of the assay.

Vandekar et al[41] established a dose response relationship with the methylcarbamate, propoxur, and the inhibition of acetylcholin-

esterase. In an attempt to establish a similar relationship with carbaryl, it was found that the 50 mg/kg *in vivo* dose appears to inhibit cholinesterase of blood and saliva to the same extent as the 200 mg/kg dose (Figure 4). It was also noted that the pattern of metabolites was dose-dependent. In saliva, B was present at the 50 mg/kg level but absent at the 200 mg/kg level. This metabolite has been identified as 5,6-dihydro-5,6-dihydoxy-1-methylcarbamate which is a cholinesterase inhibitor. Metabolite D, 1-hydroxy-5,6-dihydroxy naphthalene, has no anticholinesterase activity.[31] This change in circulating metabolites could explain the increased inhibition of cholinesterase at the 50 mg/kg level.

The uptake of Kepone into rat salivary gland slices reached a peak after about 60 minutes incubation. Incubations of gland slices with higher concentrations of Kepone did not have any significant effect on uptake or efflux. Additions of cyanide (10^{-3}M), a known inhibitor of respiration. aeration with N_2, or decrease in temperature did not alter uptake or efflux of Kepone significantly (Table 2). These data indicate a passive uptake of Kepone by the submaxillary gland.

The *in vivo* data on saliva/plasma ratios (Table 1) agree with the *in vitro* observations on uptake and efflux. Kepone is present in saliva, but to a much lesser degree than in blood. Since the saliva/plasma ratios remain less than one over the 48 hour experimental period, it can be concluded that there is no active concentrating mechanism operating in the salivary

gland cell. It may be assumed from these data that the movement of Kepone from the blood into the salivary gland and out into the oral cavity in saliva is one of passive diffusion, is not concentration-dependent, and does not have a saturable binding site in the basal membrane.

SUMMARY

Both carbaryl and Kepone enter the salivary tissue and saliva in a passive manner. Carbaryl and its metabolites appear in similar quantities in both blood and saliva, while the concentration of Kepone is three to four times lower in saliva than in blood. Kepone has no convenient biological indicator, such as anticholinesterase activity. The presence of Kepone must be detected by chemical means. Thus, it appears that blood or urine rather than saliva would provide the best indicator of exposure to Kepone. This hypothesis has been recently confirmed by Blanke et al,[26] who found only occasional trace amounts of Kepone in saliva samples taken from patients who had been chronically exposed to Kepone and exhibited significant levels of this pesticide in their blood. Saliva may prove to be a useful diagnostic fluid for compounds like carbaryl, however. The metabolites of carbaryl are excreted in saliva in a parallel fashion to plasma with a corresponding inhibition of cholinesterase activity.

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